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Role of Nucleotide P2 Receptors in Calcium Signaling and Prolactin Release in Pituitary Lactotrophs*

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Mu-Lan He, Arturo E. Gonzalez-Iglesias, and Stanko S. Stojilkovic‡

From the Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4510

Anterior pituitary cells express nucleotide-gated G protein-coupled P2 receptors (P2YRs) and cation-conducting channels (P2XRs). However, the identification of P2 receptors subtypes and their native ligands, and the distribution and function of these receptors within the secretory and non-secretory pituitary cells has been incompletely characterized. The focus in this study was on lactotroph subpopulation of cells. ATP and ADP, but not UTP and UDP, triggered calcium signaling in a majority (85%) of lactotrophs and prolactin release in mixed pituitary cells. Consistent with the role of P2 receptors in signaling and secretion, the actions of ATP and ADP were abolished in the presence of apyrase, an ectonucleotidase. Transcripts for G_q-coupled calcium-mobilizing $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$, as well as G_i-coupled $P2Y_{12}R$, were identified in mixed anterior pituitary cells. The ligand-selectivity profile of calcium mobilization-dependent signaling and prolactin secretion and the blockade of these responses by pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulphonic acid indicated that $P2Y_1R$ mediates the stimulatory action of ATP and ADP. Within the channels expressed in anterior pituitary ($P2X_2R$, $P2X_3R$, $P2X_4R$, and $P2X_7R$), the $P2X_4R$ subtype provides a major pathway for calcium influx-dependent signaling and prolactin secretion. This conclusion was based on comparison of native to recombinant channels with respect to their ligand preference, sensitivity to pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulphonic acid, and the rates of calcium signal desensitization.

Purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) are not only important intracellular molecules, but also function as extracellular messengers by activating distinct plasma membrane receptors. These receptors were termed "purinergic" in the seventies and belong to two groups: P1 receptors (P1Rs)[1] that are activated by adenosine and P2 receptors (P2Rs) that recognize mainly ADP, ATP, UDP, and UTP. Four subtypes comprise the P1R family of G protein-coupled receptors, termed A_1, A_{2A}, A_{2B}, and A_3, whereas P2Rs are composed of two families: the ligand-gated channels (P2XR) and the G protein-coupled receptors (P2YR). To date, seven functional channels ($P2X_1$ to $P2X_7$) and six receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$) have been identified in mammals (1–3). P2Rs are expressed in a variety of non-excitable and excitable tissues. Three lines of previously published evidence also indicate that an extracellular nucleotide-controlled signaling system is expressed and operative in anterior pituitary cells. First, ATP is co-stored with hormones in secretory vesicles and co-secreted by anterior pituitary cells (4, 5). Second, P1Rs, P2YRs, and P2XRs are expressed in these cells and their activation leads to amplification of calcium signals and secretion triggered by hypothalamic neurohormones (6–8). Third, the autocrine/paracrine actions of ATP are controlled by ectonucleotidases (4). These enzymes degrade extracellular ATP in a sequential manner to adenosine (9) and thus provide a pathway for activation of P1Rs.

The expression of P1R subtypes in pituitary cells, their distribution within the secretory and non-secretory pituitary cells, and their physiological roles are relatively well characterized (7, 8). A substantial progress was also made in characterizing the P2R signaling pathway in pituitary. The presence of P2YRs was initially observed in a mixed population of sheep pituitary cells (10, 11). Rat pituitary cells also express P2YRs and their activation by ATP is associated with an elevation in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) due to release of calcium from intracellular stores (12–14). Molecular cloning and functional characterization of rat P2YRs in the pituitary revealed the expression of $P2Y_2R$ with the pharmacological profile resembling the one observed in sheep pituitary cells (15). More recent studies have also suggested the presence of P2XRs in normal and immortalized pituitary cells (4, 16, 17). Initially, the mRNA messages for $P2X_{2a}R$ and its spliced form, $P2X_{2b}R$, were identified in pituitary cells (18–20). This was followed by discovery of $P2X_3R$, $P2X_4R$, and $P2X_7R$ in these cells (21).

Several important questions regarding the functional operation of nucleotide receptor signaling system in pituitary have not been addressed. For example, in contrast to P2XRs (21), the expression of P2YR subtypes and their distribution within the secretory and non-secretory pituitary cells has not been studied. The characterization of calcium signaling by P2Rs other than $P2Y_2R$ (15) is at a preliminary stage, as well as the nature of relationship between plasma membrane current and calcium signaling. The recombinant P2XRs expressed in an excitable cell generate global calcium signals (22), suggesting that nuclear and cytoplasmic cellular functions, including exocytosis, should be affected. Yet, only limited information is available about the relevance of nucleotide receptor-mediated calcium signaling in secretion, and their potential role in other cellular functions in pituitary cells has not been studied. Finally, the co-expression of several receptor subtypes, as well as ectonucle-

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‡ To whom correspondence should be addressed: Section on Cellular Signaling, ERRB, NICHD, NIH, Bldg. 49, Room 6A-36, 49 Convent Dr., Bethesda, MD 20892-4510. Tel.: 301-496-1638; Fax: 301-594-7031; E-mail: stankos@helix.nih.gov.

[1] The abbreviations used are: P1R, adenosine receptor; P2R, nucleotide receptor; PRL, prolactin; TRH, thyrotrophin releasing hormone; P2XR, nucleotide receptor-channel; P2YR, G protein-coupled nucleotide receptors; αβ-meATP, αβ-methylene-ATP; BzATP, 3′-O-(4-benzoyl)benzoyl-ATP; 2-MeSATP, 2-methylthio-ATP; PPADS, pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulphonic acid.

otidases in pituitary cells raise the question of which receptor family (P2YR, P2XRs, and/or P1Rs) and which signaling molecules (adenosine, ADP, ATP, UDP, and/or UTP) are critical for signaling and secretory actions in a particular cell type.

The focus in this study was on expression of P2YRs in anterior pituitary cells and role of both P2YRs and P2XRs in calcium signaling and secretion in pituitary lactotrophs. We screened the expression of P2YRs in pituitary tissue and cultured pituitary cells. To analyze their role in calcium signaling and PRL release, we bathed cells in calcium-deficient medium and defined the receptor pharmacological profiles using intracellular calcium and PRL measurements. To analyze the potential participation of P2XRs in calcium influx and secretion, experiments were also performed with cells bathed in calcium-containing medium, as well as in cells treated with thapsigargin, a blocker of endoplasmic reticulum Ca^{2+}-ATPase (23). Finally, the pharmacological profiles of recombinant P2XRs were compared with those observed in secretory anterior pituitary cells. The results of these investigations indicated a complex pattern of P2YR expression in pituitary cells and revealed that calcium-mobilizing $P2Y_1R$ and cation-conducting $P2X_4R$ play a major role in ATP- and ADP-induced calcium signaling and PRL release.

MATERIALS AND METHODS

Cell Cultures—Experiments were performed in anterior pituitary cells from normal postpubertal female Sprague-Dawley rats obtained from Taconic Farm (Germantown, NY) and immortalized GnRH-secreting GT1 cells. Pituitary cells were dispersed as described previously (4) and cultured as mixed cells or enriched lactotrophs in medium 199 containing Earle's salts (Invitrogen), sodium bicarbonate, 10% heat-inactivated horse serum (Invitrogen), and penicillin (100 units/ml) and streptomycin (100 μg/ml). A two-stage Percoll discontinuous density gradient procedure (4) was used to obtain enriched lactotrophs and their further identification in single cell calcium studies was done by the addition of TRH (Peninsula Laboratories, Belmont, CA) at the end of recording.

For expression of recombinant P2XRs, GT1 cells were selected because they do not express native P2Rs and, as pituitary cells, exhibit spontaneous electrical activity (22, 24). GT1 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1), containing 10% (v/v) heat-inactivated fetal bovine serum and 100 μg/ml Gentamicin (Invitrogen). Procedures for transient transfection in GT1 cells were performed as described (18). Briefly, cells were plated on 25 mm coverslips coated with poly-L-lysine (0.01% w/v; Sigma) at a density of 7.5×10^4 cell per 35 mm dish and allowed to grow for 24 h. For each dish of cells, transient transfection of expression constructs was conducted using 1 μg of DNA and 7 μl of LipofectAMINE 2000 reagent (Invitrogen) in 3 ml of serum-free Opti-MEM. After 6 h of incubation, the transfection mixture was replaced with normal culture medium. Cells were subjected to experiments 24 h after transfection.

Pituitary and GT1 cells were stimulated with ATP, ADP, $\alpha\beta$-methylene-ATP ($\alpha\beta$-meATP), 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP), and UDP (all from Sigma), as well as with ATPγS, 2-methylthio-ATP (2-MeSATP), UTP, and pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulphonic acid (PPADS) (Calbiochem, San Diego, CA). To increase ectonucleotidase activity, cells were treated with apyrase (Grade I, Sigma).

Single Cell Intracellular Calcium Measurements—For $[Ca^{2+}]_i$ measurements, cells were loaded with 1 μM Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) in modified Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 0.7 mM $MgSO_4$, 1.8 g/l glucose, and 15 mM HEPES, pH 7.4) for 60 min at room temperature. After dye loading, cells were incubated in modified Krebs-Ringer buffer and kept in the dark for at least 30 min before single-cell $[Ca^{2+}]_i$ measurement. Recordings were done in calcium-containing medium (free calcium 1.26 mM) and calcium-deficient medium (about 100 nM free calcium, adjusted by addition of EGTA). To exclude calcium mobilization from intracellular pool, cells were pretreated with 1 μM thapsigargin (RBI, Natick, MA) for at least 20 min. Coverslips with cells were mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). The dynamic changes of $[Ca^{2+}]_i$ were examined under a 40× oil immersion objective during exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 520 nm was recorded. The ratio of light intensities, F_{340}/F_{380}, which reflects changes in $[Ca^{2+}]_i$, was simultaneously followed in several single cells at rate of about 1 point/s.

Measurements of PRL and ATP—Hormone secretion and ATP content was monitored using cell column perifusion experiments, as described previously (4). Briefly, 1.2×10^7 cells were incubated with pre-swollen cytodex-1 beads in 60-mm Petri dishes for 24 h. The beads were then transferred to 0.5 ml chambers and perifused with Krebs-Ringer medium for 2.5 h at a flow rate of 0.8 ml/min and at 37 °C to establish stable basal secretion. Fractions were collected in 1 min intervals and immediately assayed for PRL and ATP contents. Primary antibody and standard for PRL assay were provided by the National Pituitary Agency and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA), ^{125}I-PRL was purchased from PerkinElmer Life Sciences and secondary antibody from Sigma. The concentration of ATP in the effluent was determined using an ATP bioluminescent assay kit (Sigma) in TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Calibration curves were constructed from measurements of standard solutions, which were diluted in the same medium as the corresponding solutions of unknown ATP concentration. Detection limit of the assay was 0.2 nM.

Reverse Transcriptase-PCR Analysis of P2YR Expression—Total RNA from rat anterior pituitary was extracted using TRIzol reagent (Invitrogen). After DNase I digestion, 2 μg of total RNA was reverse-transcribed into first-strand cDNA with oligo-dT primers and SuperScriptII reverse transcriptase (Invitrogen). The cDNAs were then amplified with different P2YR isoform-specific primer sets in the non-conserved carboxyl-terminal region. The oligonucleotide sequences of primers used for PCR amplification, with GeneBank™ accession numbers given in parentheses, are listed as $P2Y_1R$ (U22830): sense primer, 5′-GCCTGAGTTGGAAAGAAGAGGATG-3′, antisense primer, 5′-GGAAATCCAGCCGTGCCCTC-3′; $P2Y_2R$ (L46865): sense primer, 5′-CAAGAGCAGGAGCTGATCGGGTC-3′, antisense primer, 5′-CGATGGTGCGCACAGACTTG-3′; $P2Y_4R$ (Y14705): sense primer, 5′-CATGACCAGTGCAGAATCCTTG-3′, antisense primer, 5′-GGGTCGAGTCACCTTGTAAAC-3′; $P2Y_6R$ (D63665): sense primer, 5′-GGAAAAGCAGGCCGCCAGTTATG-3′, antisense primer, 5′-AGTGCGGTTGCGCTGGATGC-3′; $P2Y_{12}R$ (AF313450): sense primer, 5′-GGCAACGAAACCAAGTCACTGAG-3′, antisense primer, 5′-CACTAAAGACTGAAGCAACCC-3′.

The amplification was conducted in a Robocycler Thermal Cycler (Stratagene, La Jolla, CA) in a 50-μl reaction volume containing 1 μl of the first-strand cDNA as template, 1 unit of *Taq*DNA polymerase (Invitrogen), 0.5 μM concentration of each primer, 0.2 mM dNTP, and 1× PCR buffer (2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4). Amplification of DNA templates was initiated by a denaturation step at 94 °C for 180 s, followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 52 °C for 30 s, and extension at 72 °C for 90 s. The reaction was then terminated by a final extension step at 72 °C for 10 min. After PCR, a 10-μl aliquot of PCR products was size-fractionated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. To check for the integrity of RNA preparation, reverse transcriptase-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also conducted as an internal control using primers GAPDH-sense (5′-GGCATCCTGGGCTACACTG-3′) and GAPDH-antisense (5′-TGAGGTCCACCACCCTGTT-3′).

RESULTS

Ligand and Ectonucleotidase-dependence of PRL Release—To define the pattern of signals applied in our perifusion experiments, we added ATP to chambers with and without cells, collected fractions every minute, and measured ATP concentrations immediately after collection. Fig. 1*A* illustrates concentrations of ATP measured in effluents prior, during, and after the addition of 10 μM ATP. In effluents from chambers containing beads without cells, free ATP concentrations reached the steady level shortly after its addition and returned back to undetectable levels within 2 min after stopping its application. In chambers containing beads with cells, the profile of ATP content was similar as described above, but the steady levels was only about one fourth of that found in effluents from chambers without cells (Fig. 1*A*). This finding is consistent with earlier studies showing the operation of endogenous ectoATPases in pituitary cells (4). Thus, in our experimental conditions the ATP signal has a square-like type and native ectoATPases influence its amplitude but not its pattern.

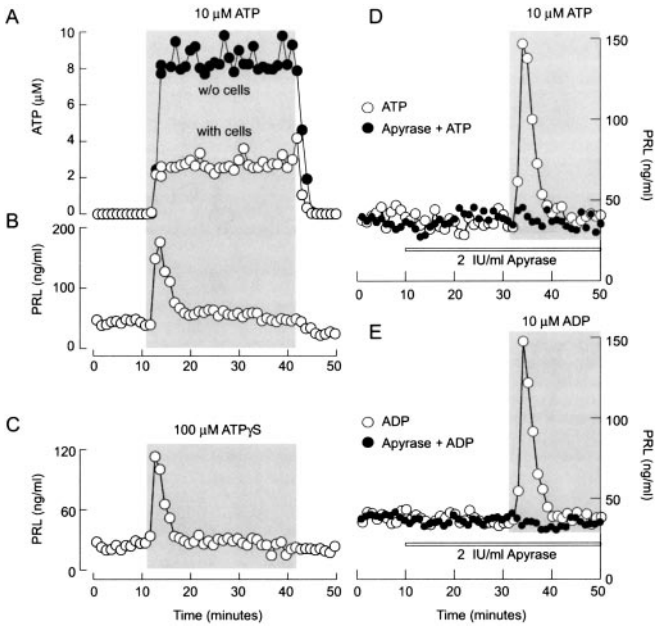

FIG. 1. **Dependence of PRL release on P2R activity.** *A* and *B*, comparison of ATP profiles and PRL release in pituitary cells perifused with calcium-containing medium. *A*, cells were perifused with 10 μM ATP and its concentration was measured in effluents from columns with (*open circles*) and without cells (*filled circles*). *B*, PRL content was measured in effluents from column with cells. *C*, effects of 100 μM ATPγS, a non-hydrolysable agonist for P2Rs, on PRL release. *D* and *E*, 2 IU/ml apyrase abolished effects of ATP (*D*) and ADP (*E*) on PRL release. *Gray areas* indicate the duration of agonist application. Cells were perifused at flow rate of 0.8 ml/min at 37 °C. In these and following figures, the PRL secretion profiles are representative from at least three independent experiments.

To examine the potential secretagogue action of ATP, we also measured PRL content in effluents from chambers with cells attached on beads. As shown in Fig. 1*B*, there was high basal PRL release prior to the addition of ATP and further increase in PRL release occurred during perifusion of cells with ATP. In contrast to the square-type ATP signal, the secretion of PRL was composed of two phases: a rapid spike response and a sustained plateau response (Fig. 1*B*). The early spike response was observed in all experiments ($n = 44$), whereas the sustained low amplitude response was only visible in some experiments. ATPγS, a non-hydrolysable agonist for majority of P2Rs (1, 25), also induced rapid and transient increase in PRL release (Fig. 1*C*).

To clarify which agonist acts as a native extracellular messenger, cells were stimulated with ATP, ADP, UTP, and UDP. As shown in Fig. 1, *D* and *E*, ATP and ADP induced comparable increase in PRL release. Furthermore, the secretory action of these two agonists was abolished in cells perifused with apyrase, an ectonucleotidase that degrades both ATP and ADP. In contrast, only a small increase in PRL release was observed in cells perifused with UTP and UDP (not shown). These results indicate that ATP and ADP, rather than their degradation products AMP and adenosine, are native P2R agonists in lactotrophs.

Agonist-induced PRL release does not provide the evidence that P2Rs are expressed in lactotrophs, because the secretory action of nucleotides could be mediated indirectly, through a paracrine factor released by another cell type, like endothelins that are known to modulate secretion in lactotrophs (26, 27). However, single cell calcium measurements revealed that 88% of TRH-responsive cells responded to ATP stimulation with an elevation in $[Ca^{2+}]_i$ (Fig. 2*A*). In a fraction of lactotrophs (76 of 614 TRH-sensitive cells) no increase in $[Ca^{2+}]_i$ was observed

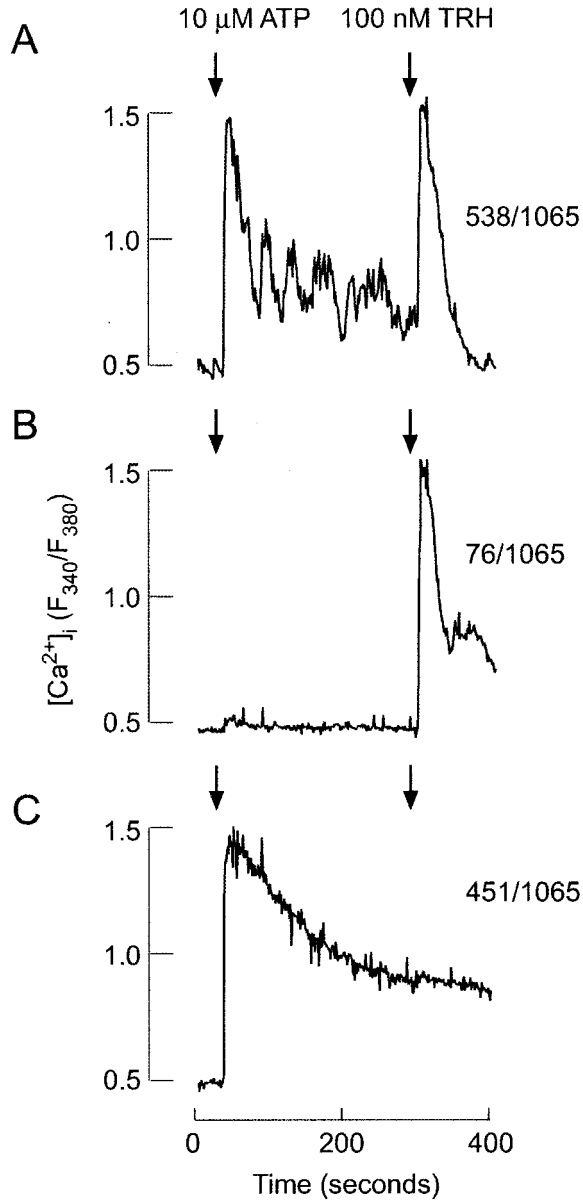

FIG. 2. **10 μM ATP induced calcium signaling in pituitary cells bathed in calcium-containing medium.** *Numerators* indicate number of cells responding to ATP and TRH (*A*), only TRH (*B*), and only ATP (*C*). *Denominators* indicate total number of cells in five independent experiments.

(Fig. 2*B*). Cells other than TRH-sensitive also responded to ATP stimulation under these conditions (Fig. 2*C*). These data clearly indicate that majority of lactotrophs express P2Rs capable of elevating the $[Ca^{2+}]_i$.

Expression of P2YRs in Pituitary Cells—In further studies, we analyzed the presence of mRNA transcripts for mammalian P2YRs in rat anterior pituitary tissue. This analysis revealed the presence of rat-specific mRNAs for $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{12}R$ (Fig. 3*A*). Functional analysis of P2YRs was further studied at single cell level. With the exception of $P2Y_{12}R$, all other receptor subtypes expressed in pituitary cells belong to family of G_q-coupled receptors, *i.e.* their activation leads to the stimulation of phospholipase C, generation of inositol 1,4,5-trisphosphate, and release of calcium from intracellular stores (28, 29). Thus, single cell calcium measurements provide a valid method for functional characterization of these receptors. To exclude the possible participation of P2XRs in calcium signaling, we stimulated mixed population of pituitary

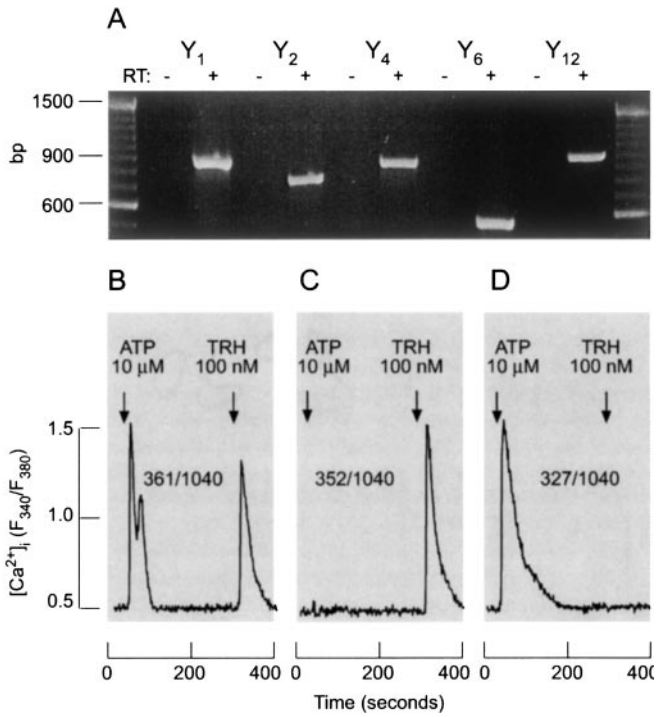

FIG. 3. **Characterization of P2YRs expressed in anterior pituitary cells.** *A*, detection of P2YR mRNA transcripts in mixed population of anterior pituitary cells. First strand cDNA synthesis reaction was performed with (+) and without (−) reverse transcriptase (*RT*). DNA markers are shown in the *first* and *last lanes*. *B–D*, 10 μM ATP-induced calcium mobilization in pituitary cells. To exclude calcium influx, cells were bathed in calcium-deficient medium (free calcium concentration was about 100 nM, adjusted by the addition of EGTA). TRH (100 nM) was added in the presence of ATP to identify lactotrophs. Traces shown are representative from five independent experiments. *Numerators* indicate number of cells responding to ATP and TRH (*B*), only TRH (*C*), and only ATP (*D*). *Denominators* indicate total number of cells.

cells with ATP in extracellular calcium-deficient medium. Fig. 3, *B* and *C*, illustrates that 51% of TRH-responsive cells also responded to ATP stimulation with a rise in $[Ca^{2+}]_i$. Similar ratio was also observed in enriched fraction of lactotrophs (Table I). However, calcium-mobilizing P2YRs were not exclusively expressed in lactotrophs; about 47% of cells other than TRH-sensitive also responded to ATP (Fig. 3*D*).

Role of P2YR in PRL Release—To clarify the potential relevance of calcium-mobilizing P2YRs in ATP-induced PRL release, pituitary cells were perifused with calcium-deficient and calcium-containing medium. As shown in Fig. 4, *A–C*, ATP and its two analog agonists, ATPγS and BzATP, induced a transient increase in PRL release in both experimental conditions. However, the peak amplitude of PRL responses in calcium-deficient medium represented only about 25% of that observed in cells perifused with calcium-containing medium.

Our enriched fractions of lactotrophs (about 70%) was not enough purified to be used for further reverse transcriptase-PCR analysis on the expression pattern of P2YRs in these cells. Thus, identification of subtypes of P2YRs responsible for extracellular calcium-independent signaling and secretion was done pharmacologically. Based on the ligand-selectivity profiles of recombinant rat P2YRs, these receptors could be separated into two groups: $P2Y_1R$ is the most sensitive to 2-MeSATP and ADP and insensitive to UTP and UDP, whereas $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$ are highly sensitive to UTP and low sensitive to 2-MeSATP (28, 29). In pituitary cells perifused in calcium-deficient medium, 2-MeSATP was more potent secretagogue than ATP, whereas UTP was practically ineffective (Fig. 4, *E–F*). The

TABLE I

Effects of 10 μM PPADS on agonist-induced calcium signaling in lactotrophs bathed in calcium-deficient (−Ca^{2+}) and calcium-containing (+Ca^{2+}) medium)

Numbers in parentheses indicate number of responders versus total number of cells. ATP + TRH, cells that respond to both agonists; TRH only, cells that respond to TRH but not ATP stimulation. Changes in F_{340}/F_{380} of 0.2 units and above were considered as specific response. ATP and TRH were added in 10 μM and 100 nM concentration, respectively.

Treatments	−Ca^{2+}		+Ca^{2+}	
	ATP + TRH	TRH only	ATP + TRH	TRH only
	%		%	
Controls	46 (61/132)	54 (71/132)	87 (160/184)	13 (24/184)
10 μM PPADS	6 (14/252)	94 (238/252)	79 (283/358)	21 (75/358)

following order reflected the ligand-selectivity profile of native P2YR controlling extracellular calcium-independent PRL release: 2-MeSATP > ATP = ADP ≫ BzATP = ATPγS ≫ UTP. Similar sensitivity was also observed in single cell calcium measurements (Table II).

The recombinant rat P2YRs also differ in their sensitivity to PPADS. This compound acts as an antagonist at $P2Y_1R$ with an IC_{50} of 5 μM, partially blocks $P2Y_6R$ at 100 μM, and does not block $P2Y_2R$ and $P2Y_4R$ (28, 29). Single cell calcium measurements in cells bathed in calcium-deficient medium revealed that ATP was unable to trigger calcium mobilization in the presence of 10 μM PPADS (Table I). The extracellular calcium-independent ATP-stimulated PRL release was also blocked by 10 μM PPADS (controls 7.5 ± 1.8 ng/ml/min, PPADS-treated 0.19 ± 0.3 ng/ml/min, basal PRL secretion subtracted). These observations support the view that $P2Y_1R$ plays a critical role in ATP-induced PRL release in cells bathed in calcium-deficient medium. The equipotency of ATP and ADP (Table II and Fig. 4*D*) further suggests that both molecules function as extracellular messengers by activating the calcium-mobilizing pathway and stimulating PRL secretion.

Role of P2XRs in Calcium Influx and PRL Release—Earlier published studies (21) have revealed the expression of $P2X_2R$, $P2X_3R$, $P2X_4R$, and $P2X_7R$ in pituitary cells. To clarify their potential role in calcium signaling and PRL release, cells were treated with different ligands in the presence of physiological extracellular calcium concentrations. To exclude calcium mobilization in agonist-induced calcium signaling, we preincubated lactotrophs with 1 μM thapsigargin for at least 20 min. In control cells, TRH-induced calcium mobilization was observed in 51 of 70 cells (73%). In thapsigargin-treated cells, the calcium-mobilizing action of TRH was blocked (not shown). After preincubation with thapsigargin, cells were treated with 100 μM agonists in the presence of physiological extracellular calcium concentrations. These results are summarized in Table III. As in experiments without depletion of intracellular calcium pool (Fig. 2), the majority of cells responded to ATP stimulation with facilitation of calcium influx. The efficacy of agonists was: 2-MeSATP > ATP > BzATP > ADP > αβ-meATP > UTP = UDP. In parallel experiments, we examined the ligand preference of receptors mediating the calcium influx-dependent PRL secretion (Fig. 5) and found that in all doses studied, the following profile: 2-MeSATP > ATP > BzATP > ADP> ATPγS ≫ αβ-meATP.

The pharmacological profiles of calcium influx-dependent signaling and PRL release provides only a partial explanation for the nature of receptors involved in these processes. αβ-meATP is a specific agonist for $P2X_3R$, acts as a partial agonist in $P2X_4R$-expressing cells, and is practically ineffective in $P2X_7R$-expressing cells (1). In our experiments, αβ-meATP raised $[Ca^{2+}]_i$ only in a small fraction of cells (Table III) and

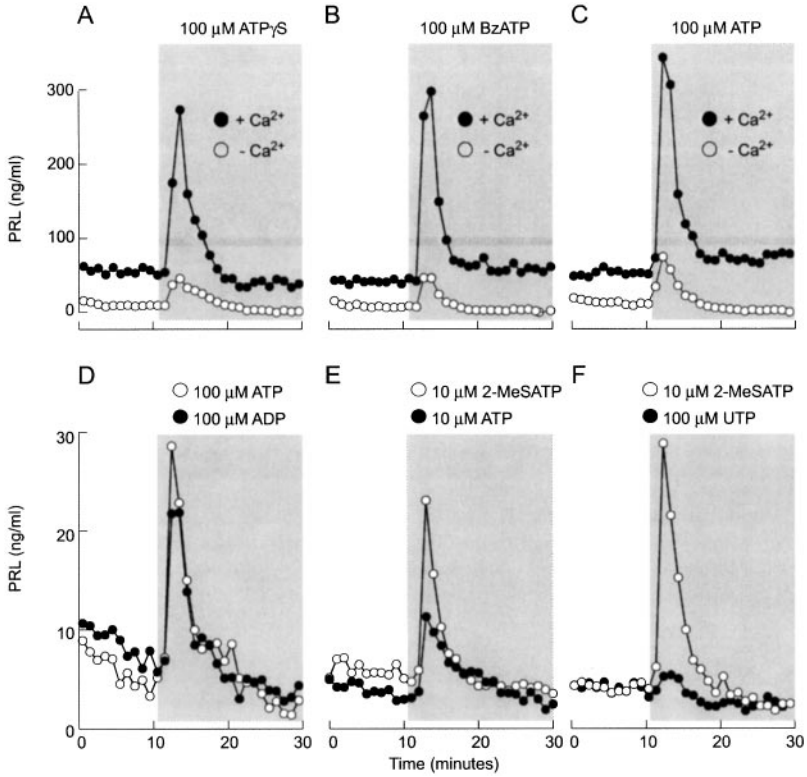

FIG. 4. **Dependence of P2R agonist-induced PRL release on extracellular and intracellular calcium.** *A–C*, cells were perifused with calcium-containing medium (1.26 mM; *filled circles*) and calcium-deficient medium (about 100 nM; *open circles*) and stimulated with 100 μM ATPγS (*A*), BzATP (*B*), and ATP (*C*). *Gray areas* indicate the duration of agonist application. Notice a difference in basal PRL release in calcium-containing *versus* calcium-deficient medium. For clarity, the first 10 min of perifusion with calcium-containing and -deficient medium are not shown. *D–F*, ligand-specificity of extracellular calcium-independent PRL release. Comparison of the effects of ATP and ADP (*D*), 2-MeSATP and ATP (*E*), and 2-MeSATP and UTP (*F*) on PRL release in cells perifused with calcium-deficient medium.

TABLE II
Effects of P2R agonists on calcium signaling in lactotrophs bathed in calcium-deficient medium

ATP and TRH, cells that respond to both agonists; TRH only, cells that respond to TRH but not ATP stimulation. Changes in F_{340}/F_{380} of 0.2 units and above were considered as specific response. Purinergic agonists were added in 100 μM and TRH in 100 nM concentrations.

Treatment	Agonist and TRH	TRH only
	%	
2-MeSATP	55 (160/289)	45 (129/289)
ADP	51 (32/62)	49 (30/62)
ATP	47 (50/106)	53 (56/106)
BzATP	26 (10/38)	74 (28/38)
αβ-MeATP	13 (8/62)	87 (54/62)
UDP	13 (10/75)	87 (65/75)
UTP	11 (7/62)	89 (55/62)

TABLE III
Effects of P2R agonists on calcium influx in purified lactotrophs bathed in calcium-containing medium

Responders, percentage of TRH-sensitive cells that responded to purinergic agonists. To exclude agonist-induced calcium mobilization, cells were pretreated with 1 μM thapsigargin for at least 20 minutes. Changes in F_{340}/F_{380} of 0.2 units and above were considered as specific response.

Agonists (100 μM)	Responders
	%
2-MeSATP	75 (120/159))
ATP	74 (103/138)
BzATP	72 (129/179)
ADP	36 (64/180)
αβ-meATP	14 (36/256)
UTP	6 (9/144)
UDP	4 (7/172)

slightly increased PRL secretion when added in 100 μM concentration (Fig. 5), arguing against the major role of $P2X_3R$ in these processes. Furthermore, recombinant rat $P2X_3R$, $P2X_2R$, and their heteromers are highly sensitive to PPADS (2). On the other hand, in enriched lactotrophs bathed in calcium-containing medium, ATP-induced calcium signaling was affected by PPADS only in a small fraction of cells (Table I). Similarly, the extracellular calcium-dependent PRL secretion was only partially affected by PPADS (controls 802 ± 103 ng/ml/10 min, PPADS treated = 605 ± 92 ng/ml/10 min), indicating that PPADS-insensitive P2XRs play a major role in agonist-induced calcium influx and PRL secretion. Among the channels expressed in pituitary, $P2X_4R$ and maybe $P2X_7R$ satisfy this requirement (1).

Recombinant versus Native P2XRs—To dissociate which of two receptors, $P2X_4R$ or $P2X_7R$, is involved in extracellular calcium-dependent signaling and secretion, we expressed recombinant rat receptors in GT1 cells, analyzed their pharmacological profiles, and compared them with those observed in pituitary cells. Fig. 6 illustrates dose-dependent effects of BzATP, 2-MeSATP, ATP, and ADP on the amplitude of calcium response in cells expressing $P2X_4R$ and $P2X_7R$. Both receptors exhibited comparable sensitivities to BzATP (Fig. 6*A*). On the other hand, $P2X_4R$ and $P2X_7R$ differed in their sensitivities to ATP (Fig. 6*B*), 2-MeSATP (*C*) and ADP (*D*), all consistent with the hypothesis that $P2X_4R$ plays a major role in calcium influx in lactotrophs. For example, the estimated EC_{50} for ATP in $P2X_4R$-expressing cells was 1 μM concentration, a concentration that was practically ineffective in $P2X_7R$-expressing cells, but triggered calcium signals in a majority of lactotrophs (Fig. 7) and induced a significant increase in PRL release in perifused pituitary cells (Fig. 5). Also, ADP induced no increase in calcium influx in $P2X_7R$-expressing cells, whereas $P2X_4R$-expressing cells show typical dose-response, with calculated EC_{50} of 100 μM (Fig. 6*D*), a concentration that triggered calcium influx in a significant fraction of pituitary cells (Table III). Typical profiles of calcium signals in lactotrophs stimulated with ATP and ADP are shown in Fig. 7. A non-oscillatory pattern of calcium signaling was only observed when lactotrophs were stimulated with high (100 μM) ATP concentrations (Fig. 7, *left panel*). In response to lower concentrations of ATP, cells usually showed initiation of calcium transients, or increase in the amplitude of transients (Fig. 7, *central panel*).

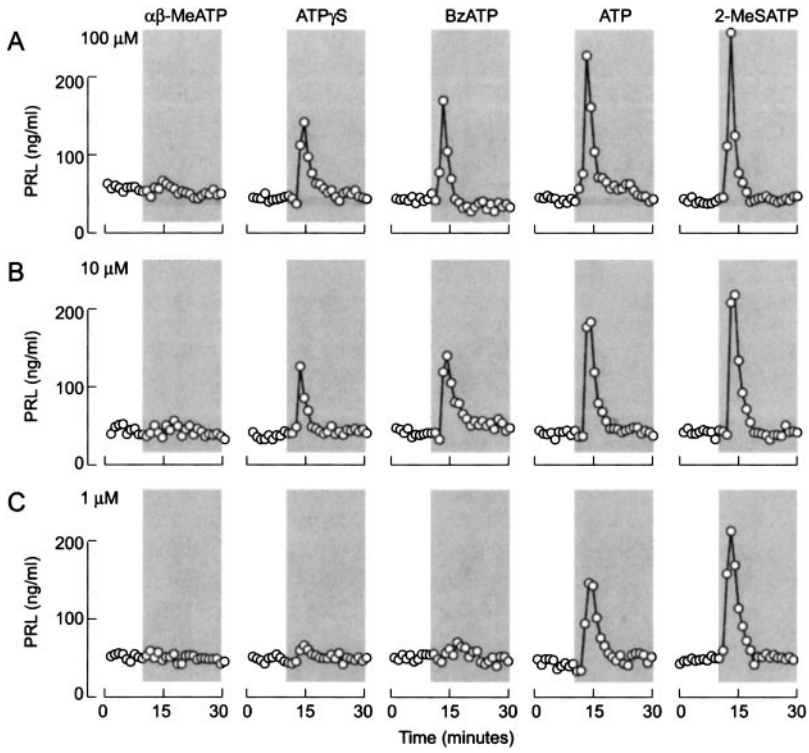

FIG. 5. **Concentration-dependent effects of P2R ligands on PRL release in pituitary cells perifused with calcium-containing medium.** *Gray areas* indicate the duration of agonist application. Ligands were added in the following concentrations: 100 μM (*A*), 10 μM (*B*), and 1 μM (*C*).

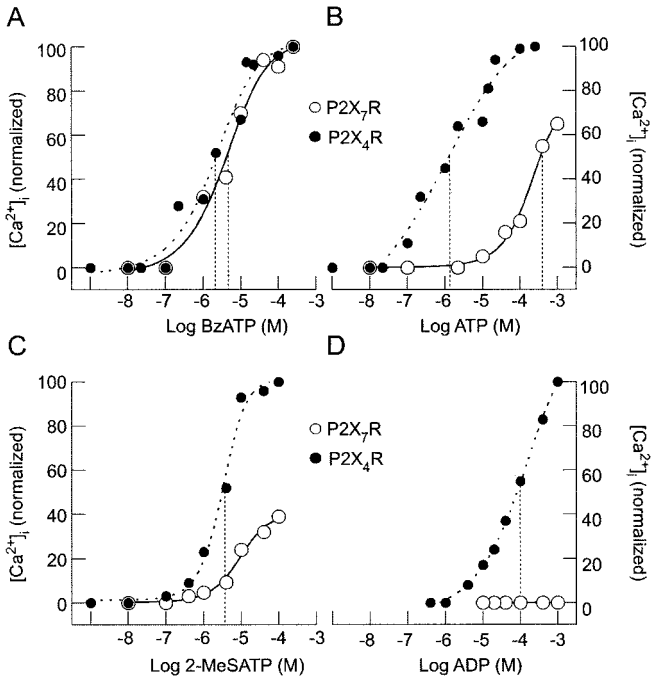

FIG. 6. **Concentration dependence of agonist-induced peak calcium response in GT1 cells expressing homomeric $P2X_4R$ (*closed circles*) and $P2X_7R$ (*open circles*).** Data shown are means derived from three to seven experiments per dose, each done in at least 15 single cells. S.E. values were within 10%. *Dotted vertical lines* indicate the calculated EC_{50} values.

Consistent with a lower potency of ADP for $P2X_4R$, the majority of lactotrophs responded to 100 μM ADP with calcium transients (Fig. 7, *right panel*).

In further experiments, we compared the profiles of calcium signals in pituitary cells and GT1 cells expressing recombinant $P2X_4R$ and $P2X_7R$. To exclude participation of P2YR in calcium signaling, pituitary cells were pretreated with thapsigargin. As shown in Fig. 8, $P2X_7R$ desensitized slowly, whereas $P2X_4R$ desensitized with a moderate rate. Native P2XR in TRH-sensitive pituitary cells desensitized with a rate that was comparable with that observed in GT1 cell expressing $P2X_4R$. These results further support the view that $P2X_4R$ provides the major pathway for calcium influx-dependent signaling and secretion in lactotrophs.

DISCUSSION

To date, four functional P1Rs and thirteen functional P2Rs and several spliced forms of these receptors have been identified in mammals (1, 2). Three of P1 receptor subtypes, A_1, A_{2A}, and A_{2B}, are also expressed in anterior pituitary cells (8). The first P2 receptor identified in anterior pituitary was $P2Y_2R$ (15). This was followed by discovery of $P2X_{2a}R$ and its spliced form $P2X_{2b}R$ in somatotrophs and gonadotrophs, and $P2X_3R$, $P2X_4R$, and $P2X_7R$ in other pituitary cell types (18, 20, 21). Here we show the presence of transcripts for four additional members of P2YRs: $P2Y_1$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$. Thus, from seventeen known nucleotide receptors, twelve of them are probably expressed in rat anterior pituitary, whereas the status of $P2Y_{11}R$ requires further attention. The finding that non-secretory anterior pituitary cells as well as neuronal and intermediate lobes of pituitary also express functional P1/P2 receptors (30–32) further adds to the complexity of this signaling system in pituitary. In that respect, only brain expresses more subtypes of these receptors. Such complexity in the expression of P1/P2 receptors suggests the potential relevance of nucleotide-receptor signaling pathways in pituitary cell functions.

The focus in this study was on lactotroph fraction of secretory anterior pituitary cells. Earlier studies have revealed that lactotrophs and GH_3 immortalized cells express G_i-coupled A1 receptors (8, 33), non-identified calcium-mobilizing P2YR (12), and probably several P2XR subtypes (21, 25). The majority of studies on purinergic control of PRL secretion were done with P1 receptor agonists and antagonists (reviewed in Refs. 7 and 8). Only one report addresses the potential role of P2 receptors on PRL secretion; in a study with dispersed pituitary cells the authors identified lactotrophs by the reverse hemolytic plaque assay and showed

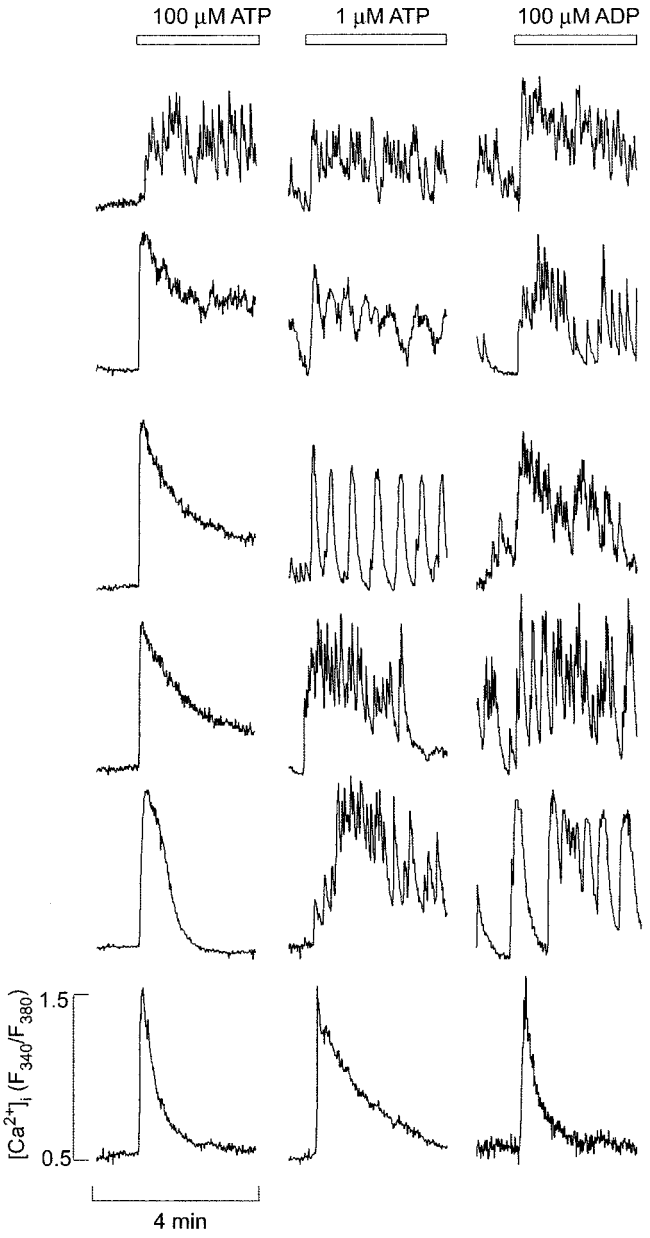

FIG. 7. **Comparison of the effects of ATP and ADP on calcium signaling patterns in lactotrophs.** Traces shown are representative from cells stimulated with 100 μM ATP (*left panel*), 1 μM ATP (*central panel*), and 100 μM ADP (*right panel*). Cells were pretreated with 1 μM thapsigargin 20 min before the addition of agonist. *Bars* indicate the duration of stimuli.

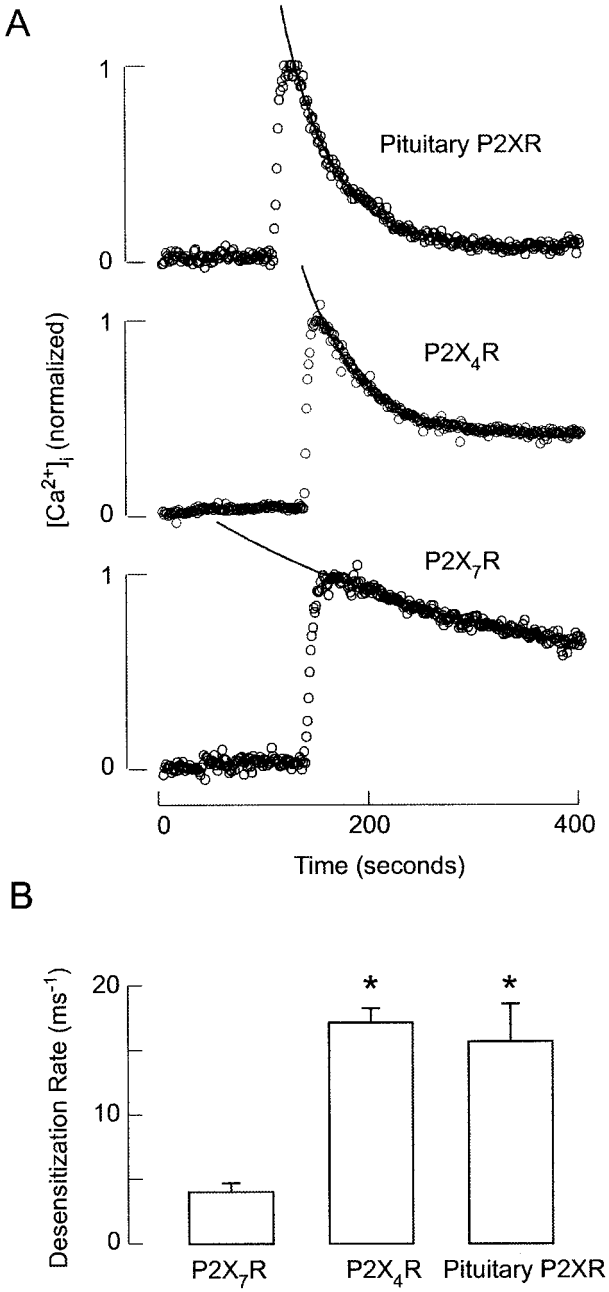

FIG. 8. **Patterns of calcium signal desensitization by native and recombinant P2XRs.** *A*, representative traces of calcium responses in cells stimulated with 100 μM ATP. Experimental records are shown by *open circles* (mean values from at least 15 traces in representative experiments), and fitted curves are shown by *full lines*. A single exponential function was sufficient to describe the desensitization rates. The fitted function is extrapolated for clarity. Pituitary cells were pretreated with 1 μM thapsigargin. Recombinant channels were expressed in GT1 cells. *B*, mean values of rates of receptor desensitization, with N of 7 to 9 experiments per receptor. *, $p < 0.01$ *versus* $P2X_7R$.

by this single cell secretory assay that ATP enhances basal and TRH-stimulated PRL release (34). Here we confirmed this finding in perifused pituitary cells.

The ability of ATP to increase PRL secretion does not necessarily indicate that ATP acts as an extracellular messenger. As we show here, there was a rapid degradation of ATP by perifused pituitary cells, suggesting that activation of ADP-sensitive P2 receptors and/or adenosine-activated P1Rs could account for this action. Consistent with the first hypothesis, ADP was also able to trigger calcium signals and PRL secretion in lactotrophs. On the other hand, two observations argued against the hypothesis that the secretory action of ATP and ADP were mediated by P1 receptors. First, ATPγS, which is a non-hydrolysable agonist for P2Rs (1, 25), also dose-dependently stimulated PRL release in calcium-containing and -deficient medium, and the pattern of secretion was highly comparable with that observed during ATP stimulation. Second, the addition of apyrase, an ectoATPase and ADPase, abolished ATP-induced secretion.

The finding that pituitary cells express five subtypes of P2YRs and four subtypes of P2XRs raised the question of which receptors are involved in control of exocytosis in lactotrophs. Our results support the view that members from both families, P2YR and P2XR, participate in ATP-stimulated PRL secretion. The relevance of P2YRs in PRL release was confirmed in experiments with calcium-deficient medium. However, the im-

portance of previously characterized pituitary $P2Y_2R$ subtype in this process was questioned in the early stage of our experiments by finding that UTP and UDP were practically unable to stimulate PRL release in calcium-deficient medium. That prompted us to search for functional expression of other members of G protein-coupled receptors. The ligand-selectivity profile of extracellular calcium-independent calcium signaling and secretion and the PPADS sensitivity of these responses were consistent with the role of $P2Y_1R$ in these processes. ADP and ATP are equipotent in activating this receptor, further confirming that both molecules act as extracellular messengers.

Earlier studies have indicated the presence of four subtypes of P2XRs in pituitary cells: $P2X_2R$, $P2X_3R$, $P2X_4R$, and $P2X_7R$ (21). The rapid inactivation of $P2X_3R$ (2), which limits calcium influx (22), argues against the potential role of these channels in stimulus-secretion coupling. In accordance with this, $\alpha\beta$-meATP, a specific $P2X_1R$ and $P2X_3R$ agonist, was unable to trigger PRL secretion. The PPADS insensitivity of extracellular calcium-dependent signaling and secretion also disputes a role for $P2X_3R$, as well as $P2X_2R$ and their heteromers in ATP-induced signaling and secretion. On the other hand, two other receptors expressed in pituitary cells, $P2X_4R$ and $P2X_7R$, exhibit low sensitivity to PPADS when expressed as homomers (2).

Two lines of evidence support the view that $P2X_4R$ rather than $P2X_7R$ plays a major role in calcium-influx-dependent signaling and secretion in lactotrophs. Desensitization of homomeric $P2X_4R$ is intermediate (2), but this receptor is able to generate global transient calcium signals (22) needed for activation of secretory pathway. On the other hand, homomeric $P2X_7R$ is a non-desensitizing channel (2), and their activation leads to the generation of long-lasting global calcium signals during prolonged agonist stimulation (22). Native P2XRs in lactotrophs desensitized in a manner highly comparable with that observed in cells expressing homomeric $P2X_4R$. In parallel to calcium signaling, agonist-induced PRL secretion was rapidly activated and gradually desensitized. The ligand-selectivity profile of calcium signaling and secretion in lactotrophs was highly comparable with that observed in calcium recording from cells expressing $P2X_4R$. Of all the ligands used in our experiments, those with ATP and ADP provided the strongest evidence that $P2X_7R$ do not participate in calcium signaling and secretion in lactotrophs. The EC_{50} for ATP in cells expressing recombinant $P2X_4R$ was 1 μM, a concentration that was unable to trigger calcium signaling in $P2X_7R$-expressing cells, whereas in lactotrophs, 1 μM ATP elevated the $[Ca^{2+}]_i$ in the majority of cells and stimulated PRL release. Also, ADP was able to trigger calcium-influx-dependent secretion and to activate $P2X_4R$, but not $P2X_7R$.

In general, comparison of homomeric P2XRs expressed in GT1 cells with native receptors could be misleading, because heteromultimerization of P2XR subunits frequently occurs, leading to the generation of receptors with distinct features. For example, $P2X_3R$ that is expressed in pituitary cells can make functional heteromers with several other subunits (35). However, it transfers $\alpha\beta$meATP sensitivity to these units (36) and does not make functional heteromers with two other channels expressed in pituitary cells, $P2X_4R$ and $P2X_7R$ (35). Thus, the inability of $\alpha\beta$meATP to trigger secretion argues against the role of these channels as homomers and heteromers in PRL secretion. On the other hand, $P2X_4R$ can make functional heteromers only with $P2X_5R$ and $P2X_6R$ (35), which are not expressed in pituitary cells (21), whereas $P2X_7R$ are unique among P2X channels for its inability to make heteromers (35).

In conclusion, here we show that pituitary cells express four P2YR subtypes, $P2Y_1R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{12}R$, in addition to previously characterized $P2Y_2R$. Our results further indicate that $P2Y_1R$ is also involved in control of PRL release. The pharmacological profile of extracellular calcium-dependent signaling and secretion is consistent with the major role of $P2X_4R$ in these processes. Together with the previously published finding that $P2X_2R$ are involved in control of LH release, these findings support the conclusion about the receptor- and ligand-specificity in nucleotide-controlled hormone secretion. Additional studies are needed to clarify the distribution and function of other members of P2Rs in anterior pituitary.

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